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Expressions of apoptosis-related proteins in rats with focal cerebral ischemia after *Angong Niuhuang* sticker point application[☆]

Dongshu Zhang, Maodong Fu, Chenglong Song, Caiyun Wang, Xiaochun Lin, Yuanliang Liu

College of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Abstract

In this study, we extracted and purified components in the *Angong Niuhuang* pill. Then we applied transdermal enhancers to *Angong Niuhuang* stickers by modern technology. The *Angong Niuhuang* sticker includes extracts from curcuma, berberine hydrochloride, baicalin, geniposide, borneol, and musk. *Angong Niuhuang* stickers at different point application doses (1.35, 2.7, and 5.4 g/kg) were administered to *Dazhui* (DU14), *Qihai* (RN6) and *Mingmen* (DU4). Rats in the different dose point application and acupuncture groups were continuously administered for 7 days. Then a middle cerebral artery occlusion model was prepared for simulating human cerebral ischemia. Twelve hours later, expressions of Bcl-2, Bax and p53 protein in hippocampal CA1 were detected with immunohistochemistry. The expression level of Bcl-2, an anti-apoptotic protein, significantly increased in the high-, medium- and low-dose point application groups and the acupuncture group, while the expression of the pro-apoptotic proteins, Bax and p53, significantly decreased compared with the middle cerebral artery occlusion rats; and the Bcl-2/Bax ratio was significantly increased. The difference was noticeable for the high-dose point application group, which showed statistical difference compared with the low-dose point application group and the acupuncture group. Our experimental findings indicate that point application with *Angong Niuhuang* stickers promotes the expression of Bcl-2, and inhibits the expressions of Bax and p53 in the hippocampal CA1 area of rats after focal cerebral ischemia. Thus, point application of *Angong Niuhuang* stickers protects brain tissues from cerebral ischemia.

Key Words

cerebral ischemia; point application; acupuncture; *Angong Niuhuang* sticker; Bcl-2; p53; Bax; apoptosis; brain injury; neural regeneration

Research Highlights

- (1) We prepared *Angong Niuhuang* stickers using transdermal enhancers with purified extracts from the *Angong Niuhuang* pill.
- (2) *Angong Niuhuang* stickers at different point application doses (1.35, 2.7, and 5.4 g/kg) were applied to *Dazhui* (DU14), *Qihai* (RN6) and *Mingmen* (DU4), to prevent cerebral ischemia.
- (3) Point application with *Angong Niuhuang* stickers promotes the expression of Bcl-2 and inhibits the expressions of Bax and p53 in the hippocampal CA1 area of rats after focal cerebral ischemia.
- (4) The *Angong Niuhuang* sticker is equivalent to electric acupuncture in regulating apoptosis related proteins in the hippocampus of cerebral ischemic rats.

Abbreviation

MCAO, middle cerebral artery occlusion

Dongshu Zhang[☆], M.D.,
Lecturer, College of Traditional Chinese Medicine,
Southern Medical University,
Guangzhou 510515,
Guangdong Province, China

Corresponding author:
Dongshu Zhang, College of Traditional Chinese Medicine,
Southern Medical University, Guangzhou 510515,
Guangdong Province, China
nyzds@sina.com

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INTRODUCTION

Acute cerebral ischemia is one of the most common cerebrovascular diseases. However, there are few effectively preventive measures. At present, point application as a route of administration is a complex therapy combining points and meridians with drugs^[1]. Previous research has confirmed that point application can reduce the area of cerebral infarction, decrease edema and inflammation reactions around necrotic lesions, and promote the proliferation and immigration of endothelial cells, which leads to increased numbers of neurons in ischemic areas^[2].

The current drugs of point application^[3-13] in the clinic for cerebral ischemia are mostly self-made prescriptions from *Rhizoma Chuanxiong*, astragalus root, red peony root, borneol, angelica, radix salviae miltiorrhizae, leech, Radix Clematidis, cassia twig and dragon's blood. When analyzing the characteristics of the above prescriptions, we found that the component drugs promote blood circulation and remove blood stasis, expel wind and dredging collaterals, and combine with drugs for invigorating *qi*. Thus, these components improve limb function, and enhance the recovery and sequela stages of hemiplegia after cerebral ischemia. The *Angong Niu Huang* pill is a classic formula prescribed for cerebral ischemia in the clinic. We extracted and purified components in the *Angong Niu Huang* pill. Then we applied transdermal enhancers to *Angong Niu Huang* stickers using modern technology. The *Angong Niu Huang* sticker is a new preparation for transdermal absorption with traditional Chinese medicine and consists of extracts from curcuma, berberine hydrochloride, baicalin, geniposide, borneol, and musk. These components clear heat and resolve phlegm, which induces resuscitation. While the pathogenesis of coma cannot direct the *qi* of stroke, the *Angong Niu Huang* stickers are suitable for the acute stage of cerebral ischemia in the clinic.

To further study the protective effect of *Angong Niu Huang* components and elucidate its protective mechanism against cerebral ischemia, we performed experiments to determine the effect of point application on the expressions of Bcl-2, Bax and p53 proteins in the rat brain tissues with cerebral ischemia.

RESULTS

Quantitative analysis of experimental animals

A total of 48 rats were randomized into six groups ($n = 8$

in each group): sham operation group, model group (middle cerebral artery occlusion model, MCAO), acupuncture group (MCAO model + acupuncture), low-, medium- and high-dose point application groups (MCAO model + 1.35, 2.7, or 5.4 g/kg *Angong Niu Huang* sticker). All 48 rats were involved in the final analysis.

The expressions of Bcl-2, Bax and p53 proteins in the hippocampal CA1 region of cerebral ischemic rats

Immunohistochemical staining showed that, compared with the sham-operation group, the Bcl-2, Bax and p53 proteins in the hippocampus CA1 area of rats in the MCAO model group demonstrated increased expression (as stained with a brown yellow color). The positive expression of Bcl-2 and Bax was mainly located in the cytoplasm, presenting a granular distribution, with nuclear pyknosis concentrated in plasma. The positive expression of p53 was mainly located in the cell nucleus, revealing brown yellow or brown granules, with a small amount of cytoplasmic expression. Compared with the model group, the cell morphology tended to be normal in point application groups and the acupuncture group, with increased expression of Bcl-2 protein in the hippocampus CA1 area and decreased Bax and p53 protein levels (Figure 1).

Quantitative analysis of Bcl-2, Bax and p53 proteins in the hippocampal CA1 region of cerebral ischemic rats

The quantitative analysis results showed that after cerebral ischemia for 12 hours, compared with the sham operation group, the mean absorbances of Bcl-2, Bax and p53 were significantly increased in the hippocampus of rats in the model group ($P < 0.01$). The expression of Bcl-2 increased in different dose point application groups and the acupuncture group ($P < 0.01$), while the expression of Bax and p53 decreased ($P < 0.05$ or $P < 0.01$). There was no statistical difference between different dose point application groups and the acupuncture group ($P > 0.05$). Compared with the sham operation group, the ratio of Bcl-2/Bax in the model group decreased ($P < 0.05$). Compared with the model group, the ratio of Bcl-2/Bax increased in different dose point application groups and the acupuncture group ($P < 0.01$). This difference was noticeable for the high-dose point application group, which showed statistical difference compared with the low-dose point application and the acupuncture groups ($P < 0.01$; Table 1).

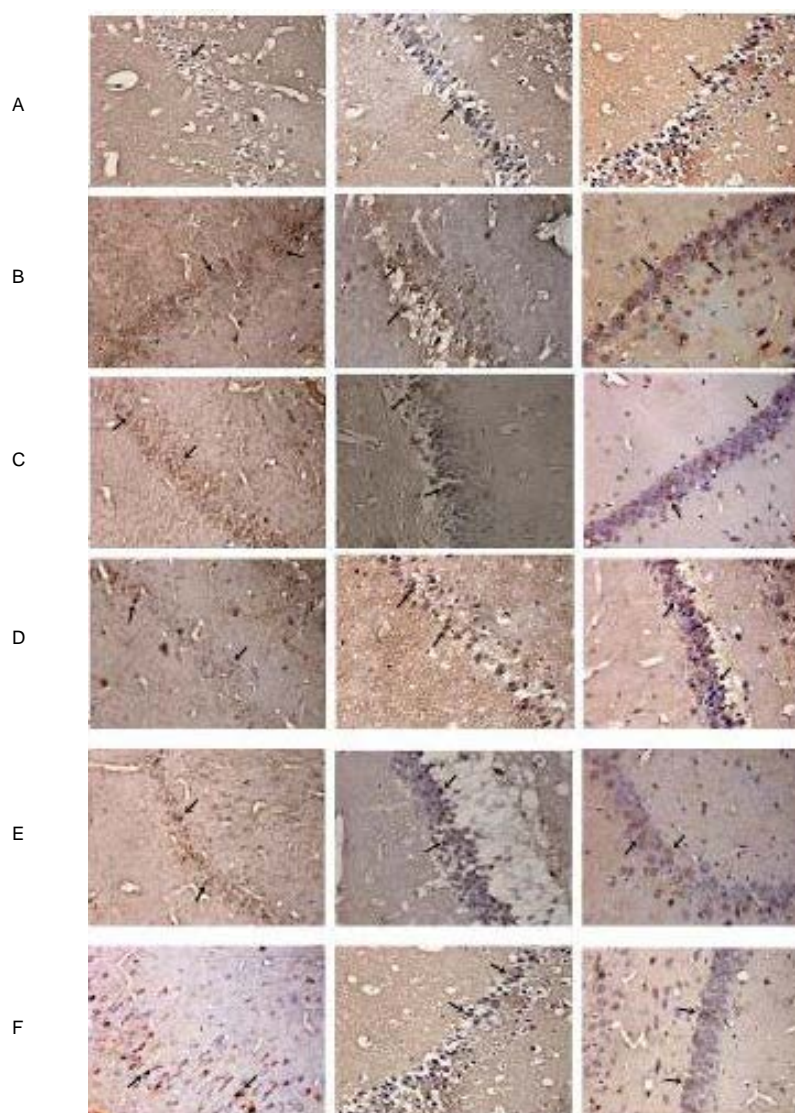


Figure 1 The expression of Bcl-2, Bax and p53 proteins in the hippocampal CA1 areas at 12 hours after cerebral ischemia (immunohistochemistry staining, optical microscope, $\times 400$). The arrows indicate the positive cells.

(A) Sham operation group; (B) model group; (C) acupuncture group; (D–F) low-, medium-, high-dose *Angong Niu Huang* sticker point application groups, respectively.

Table 1 Effects of *Angong Niu Huang* sticker point application and acupuncture on expression of Bcl-2, Bax and p53 proteins (mean absorbance) in the hippocampal CA1 areas at 12 hours after cerebral ischemia

Group	Bcl-2	Bax	Bcl-2/Bax	p53
Sham operation	0.059 \pm 0.010 ^b	0.059 \pm 0.011 ^b	1.004 \pm 0.042 ^a	0.058 \pm 0.024 ^b
Model	0.095 \pm 0.024	0.204 \pm 0.064	0.528 \pm 0.258	0.156 \pm 0.021
Acupuncture	0.188 \pm 0.037 ^b	0.140 \pm 0.047 ^a	1.291 \pm 0.176 ^{bc}	0.104 \pm 0.037 ^b
Low-dose point application	0.155 \pm 0.028 ^b	0.141 \pm 0.030 ^a	1.148 \pm 0.316 ^{bc}	0.118 \pm 0.036 ^a
Medium-dose point application	0.158 \pm 0.021 ^b	0.114 \pm 0.038 ^b	1.464 \pm 0.318 ^b	0.096 \pm 0.021 ^b
High-dose point application	0.182 \pm 0.043 ^b	0.110 \pm 0.043 ^b	1.649 \pm 0.266 ^b	0.092 \pm 0.025 ^b

The data are represented as mean \pm SD. There were eight rats in each group. ^a $P < 0.05$, ^b $P < 0.01$, vs. model group; ^c $P < 0.01$, vs. high-dose point application group (one-way analysis of variance followed Student-Newman-Keuls test). Mean absorbance = integrated absorbance/total areas.

DISCUSSION

At present, point application is being researched interna-

tionally as a route of administration. Point application is a complex therapy combining points and meridians with drugs. Point application avoids not only the “peak-valley phenomenon” found with oral administration or injected medications, but also eliminates the first-pass effect in the gastrointestinal tract which reduces side effects. In addition, the convenience of point application is easily accepted by patients, and is suitable for long-term administration, especially for the elderly and those who cannot receive oral medication or are afraid of acupuncture^[14]. We extracted and purified components in the typically prescribed *Angong Niu Huang* pill. Then we applied transdermal enhancers to *Angong Niu Huang* stickers. Long-term clinical observations showed that point application with *Angong Niu Huang* stickers on *Dazhui* (DU14), *Qihai* (RN6) and *Mingmen* (DU4) improves the neurological function deficit syndrome of patients who have suffered from ischemic stroke. There were no adverse reactions or side effects during treatment. Recently, research has been performed to elucidate the mechanisms of point application in treating cerebral ischemia at home and abroad. Related studies^[15-17] have confirmed that point application can reduce edema after cerebral ischemia, increase the activity of plasmin, resist blood coagulation and radical damage, which then protects brain tissues. Our previous research^[2] has confirmed that point application can significantly reduce the area of cerebral infarction, decrease edema and the inflammatory response around the necrotic lesions, and promote the proliferation and immigration of endothelial cells. These outcomes increase the number of neurons in ischemic areas. In recent experiments, we found that point application with *Angong Niu Huang* stickers can reduce apoptosis after cerebral ischemia and inhibit neuronal death. Our previous studies focused on the effects of point application on the expressions of Bcl-2, Bax and p53 proteins to confirm its role in regulating apoptosis after cerebral ischemia. Bcl-2, B cell lymphoma/leukemia-2, is the earliest discovered apoptosis-related gene. Bax is a new member of the Bcl-2 family of proteins. Bcl-2 and Bax are important for cell survival and apoptosis in this family^[18]. Bcl-2 interacts with Bax, but Bcl-2 is anti-apoptotic, and Bax is a pro-apoptotic protein. Their ratio determines whether or not apoptosis is initiated when the cell is stimulated^[19]. Our results indicated that there was little expression of Bcl-2 and Bax proteins in the brain tissues of rats in the sham operation group, while Bcl-2 and Bax expression increased remarkably in the hippocampus CA1 area of ischemic brain tissue in the MCAO model group, which is consistent with the literature^[20]. Our results also showed that cerebral ischemia can induce the expres-

sions of Bcl-2 and Bax. Because Bax was dominant, the increased expression of Bax significantly decreased the ratio of Bcl-2/Bax. This decreased Bcl-2/Bax ratio in turn led the cells to the apoptotic pathway, as shown previously^[21]. Point application can promote the expression of Bcl-2, and reduce the expression of Bax to levels equivalent to that in the acupuncture group. The ratio of Bcl-2/Bax in the high-dose group increased the most. A tentative inference on these results is that point application has the effects of resisting cerebral ischemia and protecting nerve tissues through the up-regulation of Bcl-2, the down-regulation of Bax, and thus, changing the ratio of Bcl-2/Bax to decrease apoptosis.

The transcription factor p53 is usually the first decision factor in the chain reaction that leads to apoptosis. The expression of p53 rapidly increases Bax expression levels and lowers the expression of Bcl-2, which accelerates apoptosis^[22]. Thus, the expression and regulation of p53 and its related genes in cerebral ischemia play an important role in neuronal apoptosis. Our results indicated that there was little expression of p53 protein in the sham operation group, while p53 levels increased greatly in the MCAO model group, which was consistent with the literature^[23]. The expression of p53 decreased in different degrees in the point application groups and the acupuncture group. There was no statistical difference between the different dose point application groups and the acupuncture group, which showed that point application reduced the apoptosis of nerve cells after cerebral ischemia by inhibiting p53. In conclusion, point application with *Angong Niu Huang* stickers can reduce neuronal apoptosis by regulating the expressions of Bcl-2, Bax and p53 proteins and their related genes.

However, the exact mechanism needs more comprehensive, systematic and intensive research to determine the effect on the related genes. For example, point application affect what cell signal transduction pathway that regulates apoptosis?

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

The experiments were performed in the Molecular Biology Laboratory and Chinese Medicine Pharmacology Laboratory at the School of Traditional Chinese Medicine, Southern Medical University in April, 2012.

Materials

Animals

Forty-eight male eight-month pathogen-free Wistar rats, weighing 250 ± 20 g, were provided by the Experimental Animal Center of the Southern Medical University, China, with the certification number of SCXK(Yue)2006-0015. The experiments were conducted after routine feeding for 7 days at 25°C and under a 12-hour lighting cycle. All experimental protocols were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[24].

Drugs

The preparation of *Angong Niuhuang* stickers have been improved by our previous clinical and experimental research (Detailed information is given in the preparation technology section). The low-dose (1.35 g/kg) given to the rats was determined by their body surface area in proportion to a regular dose for an adult. The medium-dose (2.7 g/kg) was twice as much as the low-dose while the high-dose (5.4 g/kg) was quadruple the amount of the low-dose^[25].

The drug composition consisted of extracts from curcuma, berberine hydrochloride, baicalin, geniposide, borneol, and musk.

Preparation technology:

(1) Extraction process: Curcuma, coptis, scutellaria and gardenia (Dispensary TCM in Nanfang Hospital, Lot Number of Product: 110920, 111203, 111203, 111018) were weighed to recipe quantity. Then they were heated and extracted under reflux by ethanol. The ethanol was recycled through decompression, and the extracts were dried after filtration.

(2) Matrix preparation: Gelatin, sodium carboxymethylcellulose, polyvinylpyrrolidone and sorbitol (Reagents Center of Southern Medical University) were weighed to recipe quantity. They were immersed in the proper amount of water. The water bath was heated to allow the matrix solution to swell. Then we evenly mixed sodium polyacrylate, kaolin, propylene and glycol together, and added these components into the swelling matrix solution. Finally, a polyethylene glycol solution was added, and the matrix solution was mixed for several minutes.

(3) Drug-containing matrix preparation: We mixed the matrix with azone, menthol (Reagents Center of Southern Medical University), borneol, musk (Dispensary TCM in Nanfang Hospital, Lot Number of Product: 111018, 111107) and the drug extractions. These components were stirred evenly in the matrix to obtain a gelatinous drug-containing matrix.

(4) Finished products: The drug-containing matrices

were smeared evenly on non-woven fabrics and dried in a well-ventilated place. After being covered with a protective membrane, they were divided into stickers (1 cm × 1 cm).

Methods

Model establishment

The MCAO model was prepared by Longa's method^[26].

(1) Separation: The rats were celiac anesthetized by 10% chloral hydrate (0.35 mL/kg), then fixed on their back with a cervical median incision. The common carotid artery, internal carotid artery and external carotid artery of the right side were separated and exposed.

(2) Ligation: The external carotid artery was ligated at the bifurcation of the internal carotid artery and external carotid artery.

(3) Insertion: The right common carotid artery was cut and a nylon line was inserted into the incision using the burnt blunt head-end (diameter 0.25 mm) at a depth of 18.5 ± 0.5 mm. After the middle cerebral artery was blocked at its front end, the common carotid artery was ligated with the nylon line inside. The incision was stitched layer by layer after being disinfected by gentamicin. Metronidazole powder was used outside to prevent infection. The body temperature of the rats was kept between $36.5\text{--}37.5^{\circ}\text{C}$ during the operation. After the operations, the rats were put into cages and were fed. The sham operation group received only separation and exposure of the vessel without ligation and insertion. One hour after anaesthesia of the rats, neurologic impairment scores by Longa's report^[26] were used for evaluation. Rats receiving 1–4 points were considered successful; failures were not used in these experiments.

Treatment of point application and acupuncture

The *Dazhui*, *Mingmen* and *Qihai* points of rats, as referenced in the experimental animal points in Experimental Acupuncture Science^[27], were depilated with 8% sodium sulfide in a 1 cm × 1 cm area. For each of the three doses, each point was smeared evenly with the gelatinous drug-containing matrix by gentle massage for 5 minutes. The point then was covered with rubberized fabric, uncovered after 6 hours^[28] and cleaned with clear water once a day. After continuous therapy for 7 days, the focal cerebral ischemia model was created. The rats in the acupuncture group were acupunctured by needles 25 mm long with a 0.18 mm diameter carrying electricity for 15 minutes each time^[27] (Qingdao Xinsheng G6805–type I electro-acupuncture apparatus from Qingdao Xinsheng Co., Ltd.; Hua Tuo acupuncture needle from Suzhou Medical Appliance Factory). The points and times for the acupuncture group were the same as

the point application group. The MCAO model group and the sham operation group received catching stimulation at the same time.

Detection of Bcl-2, Bax and p53 proteins by immunohistochemistry

All rats were decapitated 12 hours after the MCAO operation. Rat brain tissues were excised and dehydrated, then cut into coronal slices from 4 mm to the front of olfactory tubercle. The coronal slices then were embedded in paraffin for immunohistochemical staining. In detail, the slices were deparaffinized following routine methods, and incubated with 3% H₂O₂ deionized water at room temperature for 5–10 minutes to inactivate endogenous peroxidases. To discard excess liquid, slices were allowed to drip normal goat serum sealing fluid at room temperature for 15 minutes. Then slices were added respectively 1:100 dilution of rabbit anti-Bcl-2, Bax or p53 polyclonal antibodies (Wuhan Boster Biotechnology Limited Company, Hubei Province, China) at 4°C overnight. After washing, the slices were immersed in biotin labeled goat anti-rabbit IgG (1:1 000; Wuhan Boster Biotechnology Limited Company) for 10–15 minutes at 20–37°C. Finally, slices were incubated with Streptavidin-HRP (Wuhan Boster Biotechnology Limited Company) at 20–37°C for 10–15 minutes. 3,3-diaminobenzidin (Wuhan Boster Biotechnology Limited Company) at room temperature was used for detection. Slices were dehydrated, cleared and mounted with neutral gums. A negative control group was created using the same steps as described above, but PBS was applied instead of the first antibody. We observed and photographed the slices with a Nikon ECLIPSE Ti-S microscope (Nikon). We randomly selected 5 brain sections from each group, and analyzed Bcl-2, Bax and p53 proteins with Nikon Image Processing Software (NIS-Elements). We used 5 non-overlapping complete fields in each section with a 400 × ocular, and measured the integrated absorbance and the total area of cells in each field, before calculating the mean absorbance (integrated absorbance/total areas)^[29].

Statistical analysis

The data were presented as mean ± SD. Significant differences were determined by one-way analysis of variance and Student-Newman-Keuls test. *P* values of < 0.05 were considered significant. All statistical analysis was performed with commercially available software (SPSS 17.0; SPSS, Chicago, IL, USA).

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Author contributions: Dongshu Zhang designed the research, analyzed the data, wrote the article, processed the statistics, and managed the funds. Maodong Fu and Chenglong Song provided and integrated the data. Caiyun Wang, Xiaochun Lin and Yuanliang Liu provided technical support and materials.

Conflicts of interest: None declared.

Ethical approval: The experimental procedures were consistent with the ethical requirements established by experimental animal ethics and welfare committee of Southern Medical University in China.

REFERENCES

- [1] Zhang GG. Practical Clinical Complete External Treatments. Beijing: Chinese medicine Science and Technology Press. 1994.
- [2] Zhang DS, Lu YK, Su ZR, et al. Effects of point application on vascular endothelial growth factor expression of focal cerebral ischemia in rats. *Zhongguo Zhenjiu*. 2009;29(3): 217-221.
- [3] Niu ZD. 40 cases of stroke sequelae treated with herbs by point application. *Henan Zhongyi*. 2005;25(11):32.
- [4] Yang JH, Bie XD, Liu H. 40 cases of stroke sequela treated with chuanxiong extraction by acupoint application. *Zhongguo Zhongyi Jichu Yixue Zazhi*. 2004;10(3):62-63.
- [5] Zhang LP, Zhang HJ, Wang CY. Preparation of point transdermal absorption polyurethane sticker named Nao shuantong. *Heilongjiang Zhongyiyao*. 1996;(6):46.
- [6] Tian WL, Jiang XD, Zhao TY, et al. Effect of warming therapy with herbs applied to acupuncture points on cerebral vascular disease. *Zhongri Youhao Yiyuan Xuebao*. 1995;9(2):80-82.
- [7] Li CH, Jia M, Zhao SK. The clinical research of point application with Shuyu cream on ischemic stroke. *Zhongguo Zhongyi Jizheng*. 2010;(1):19, 81.
- [8] Li LT, Yu M, Zhao LX. The effect observation of Wengyang Tongluo sticker on ischemic stroke. *Zhongyi Waizhi Zazhi*. 2004;13(3):34-35.
- [9] Huang F. The effect observation of point application on hemiplegia after ischemic stroke. *Zhonghua Xiandai Huli Xue Zazhi*. 2008;5(7):599-601.
- [10] Zhao CY, Tong JP, Li X. The effect observation of point application combined with comprehensive rehabilitation therapy on hemiplegia after stroke. *Guangxi Yixue*. 2012;34(5):627-628.

- [11] Gu HP, Wang MF, Deng YL. Time therapy combined with point application on 40 cases of hemiplegia after stroke. *Zhongguo Zhongyi Jizheng*. 2011;20(4):662-663.
- [12] Liu NJ. Point application with Tankeyu pill on hemiplegia after stroke. *Xiandai Kangfu*. 2001;5(2):145.
- [13] Ruan WJ. Clinical observation of the curative effect of the treatment of hemiplegia after apoplexy by the combination of electroacupuncture and acupoints plaster application. Guangzhou: Guangzhou Zhongyiyao Daxue. 2011.
- [14] Bai XH. Point Application Therapy On Chronic Intractable Disease. Beijing: Science and Technology Literature Publishing House. 2001.
- [15] Yang J, Bai H, Wan H, et al. Study on effects of t-PA and PAI activity of distill material of Ligusticum Chuanxiong Plaster by point application on cerebral ischemia-reperfusion injury in rats. *Zhongguo Yiyao Xuebao*. 2004;19(9):526-527.
- [16] Yang J, Bai H, Wan H, et al. Study on protective effect of distill material of Ligusticum Chuanxiong Plaster by point application on cerebral ischemia-reperfusion injury in rats. *Zhongguo Zhongyiyao Xinxi Zazhi*. 2004;11(9):778-779.
- [17] Yang J, Bai H, Cai Y, et al. Study on effects of cerebral water content and cerebral index of distill material of Ligusticum Chuanxiong Plaster by point application on cerebral ischemia in rats. *Jiangsu Zhongyiyao*. 2005; 26(10):65-67.
- [18] Harris MH, Thompson CB. The role of the Bcl-2 family regulation of outer mitochondrial membrane permeability. *J Cell Death Differ*. 2000;7(12):1182-1191.
- [19] Liu D, Han W, Zhang D. Effects of acupuncture at different times on expressions of Bcl-2, Bax protein in rats after focal cerebral ischemia-reperfusion. *Zhonghua Zhongyiyao Xuekan*. 2007;25(3):622-624.
- [20] Yuan QL, Yang CX, Xu P, et al. Neuroprotective effects of ginsenoside Rb1 on transient cerebral ischemia in rats. *Brain Res*. 2007;1167:1-12.
- [21] Liu N, Han Y, Chen RH, et al. The protective effect of IL8 monoclonal antibody on reperfusion injury after cerebral ischemia in rats. *Zhonghua Shenjingke Zazhi*. 2005;38(7): 417-418.
- [22] Martin LJ, Kaiser A, Yu JW, et al. Injury-induced apoptosis of neurons in adult brain is mediated by p53-dependent and p53-independent pathways and requires Bax. *J Comp Neurol*. 2001;433(3):299-311.
- [23] Han B, Sun W, Sun B, et al. Effects of acupuncture on expressions of apoptosis related genes in cerebral ischemic animal model. *Xiandai Zhongyiyao*. 2005;1(1): 58-60.
- [24] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
- [25] Chen Q. Pharmacological Studies Methodology of Traditional Chinese Medicine. Beijing: People's Health Publishing House. 2011.
- [26] Longa EZ, Weinstein PR, Carlson S, et al. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989;20(1):84-91.
- [27] Li ZR. Experimental Acupuncture Science. Beijing: Chinese Medicine Press. 2010.
- [28] Kong FS. Study on the effect and mechanism of acupoint external application on airway inflammation in Passive smoking rat. Guangzhou: Guangzhou Zhongyiyao Daxue. 2008.
- [29] Li SW. Effects of loaded swimming training on apoptosis in rat kidney cells and its mechanism of regulation. Beijing: Beijing Tiyu Daxue. 2006.

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